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(58) Field of Search
Online: EPODOC, WPI, BIOSIS, MEDLINE, CAS-ONLINE, SCISEARCH, EMBASE

(54) Abstract Title
Estrogen receptor beta (ERbeta) agonists for use in cancer treatment

(57) A pharmaceutical composition for the treatment of cancer, particularly prostate cancer, comprises an ERβ agonist such as genistein or 3βAdiol. Also claimed are a method of screening compounds for use in the treatment of prostate cancer by selecting compounds which bind ER and a method of detecting the onset of cancer in a subject by measuring the subject's levels of androgen receptor. Also described is the use of a BERKO (ERβ Knockout) mouse in the study of human prostate cancer.

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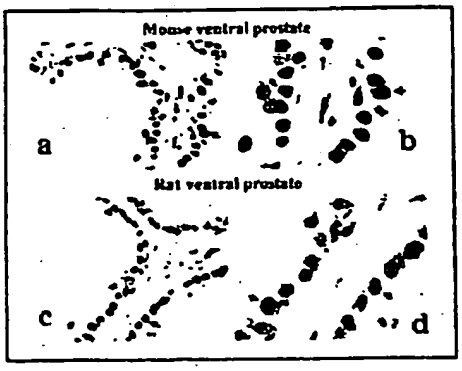
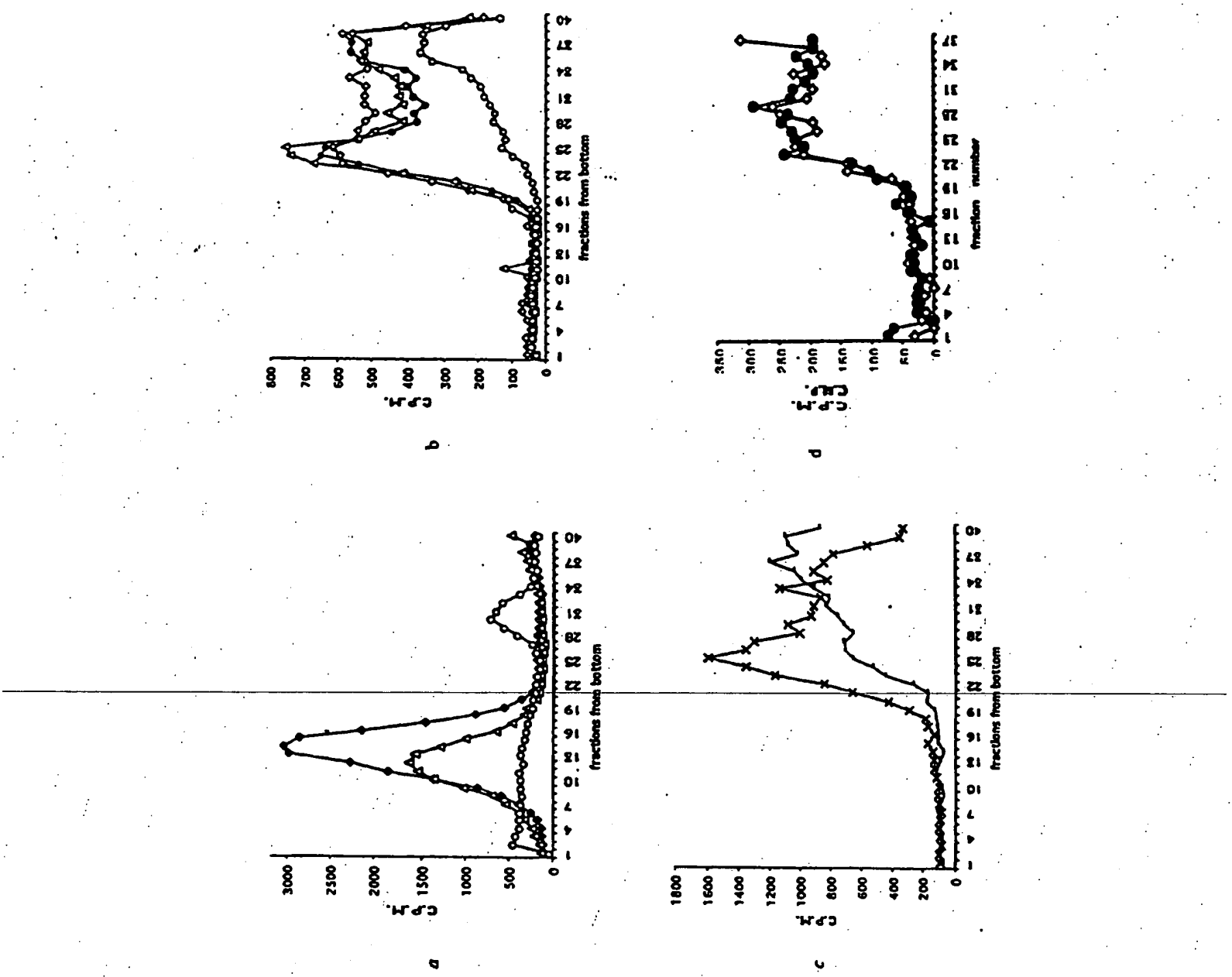


FIGURE 1



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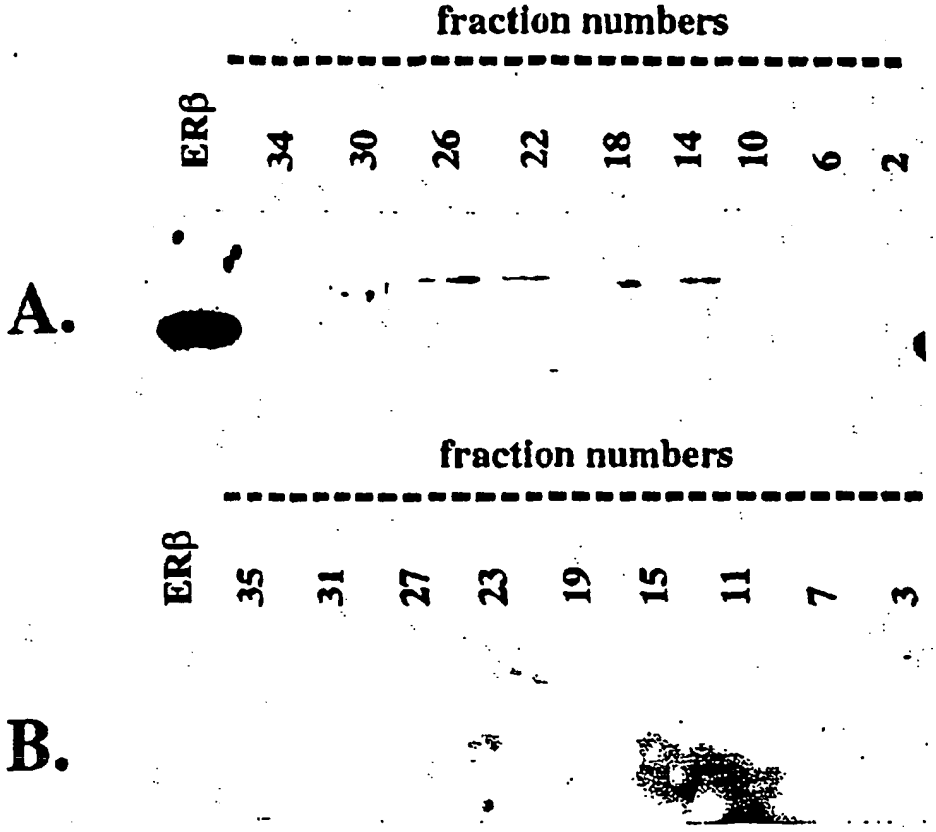

fraction numbers
ERβ
34
30
26
22
18
14
10
6
2
A.
fraction numbers
ERβ
35
31
27
23
19
15
11
7
3
B.

FIGURE 3

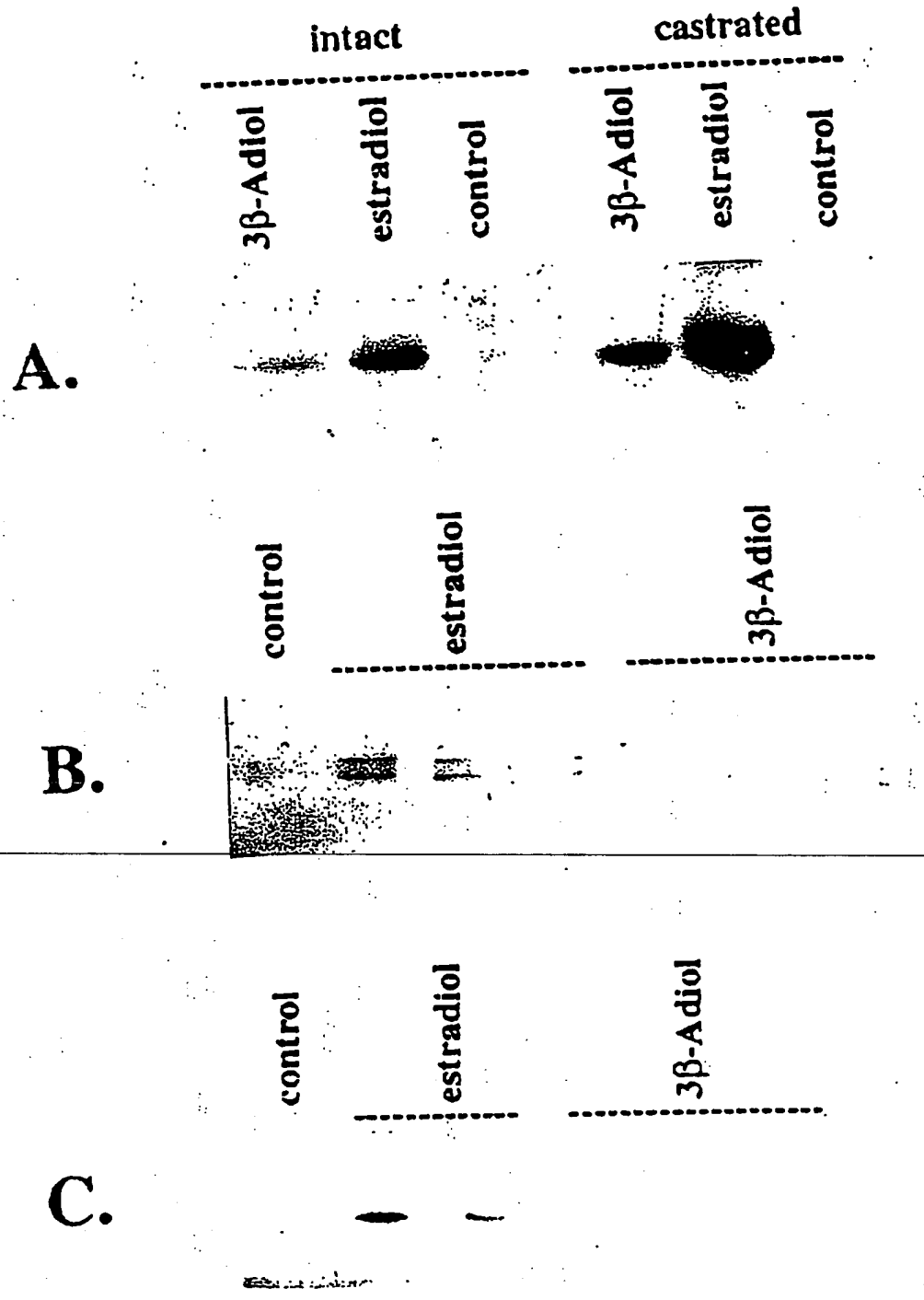
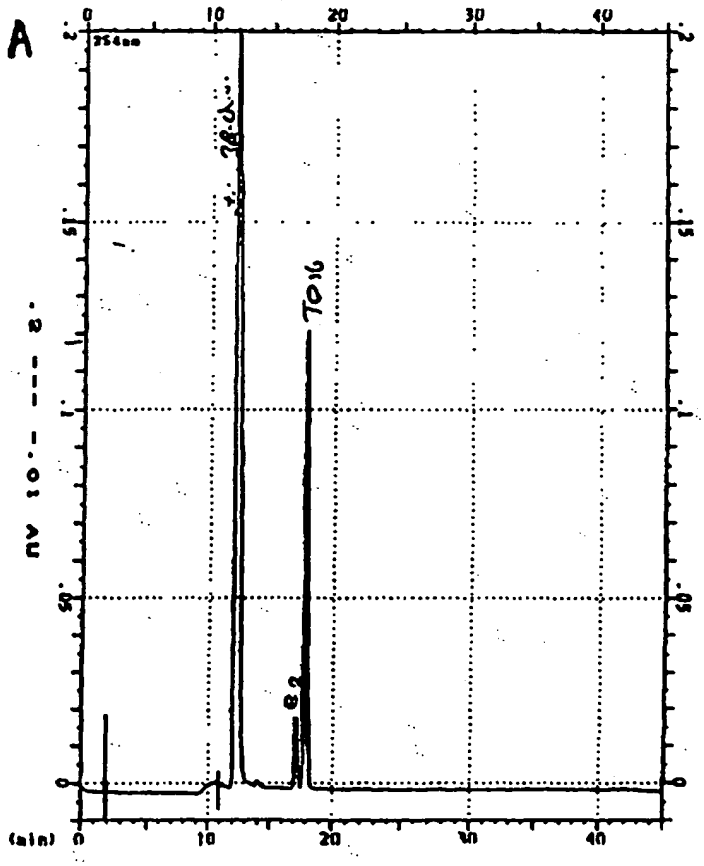

intact
castrated
3β-Adiol
estradiol
control
3β-Adiol
estradiol
control
A.
control
estradiol
3β-Adiol
B.
control
estradiol
3β-Adiol
C.

FIGURE 4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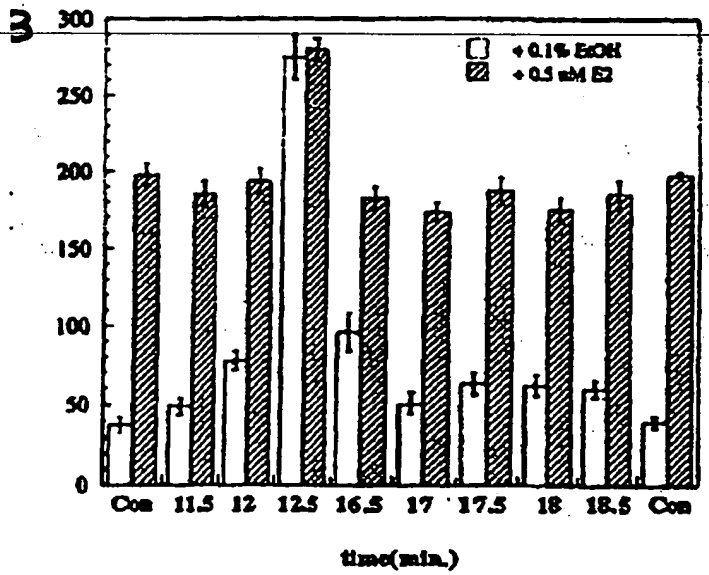
B
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200
150
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Con
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17.5
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FIGURE 5

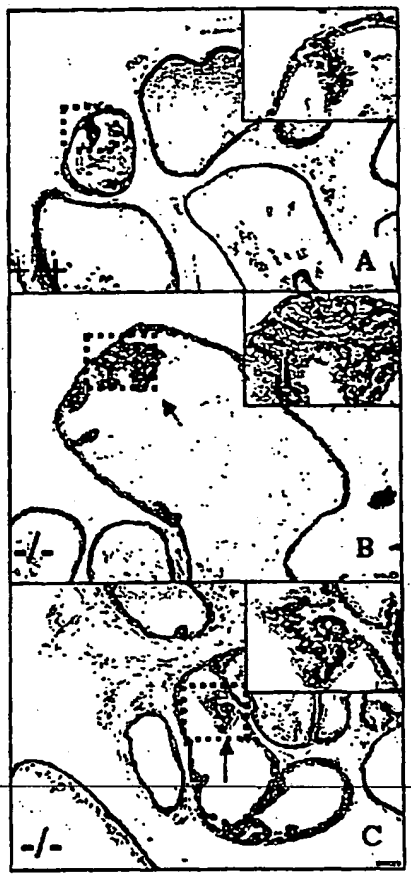

FIGURE 6

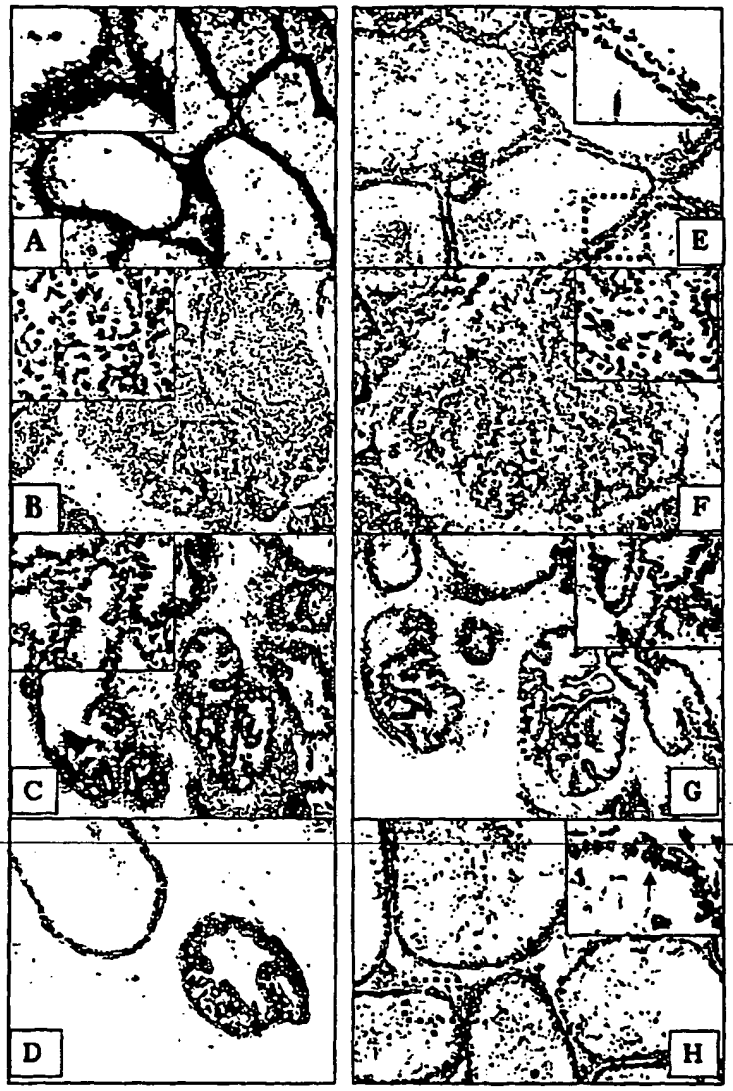

FIGURE 7

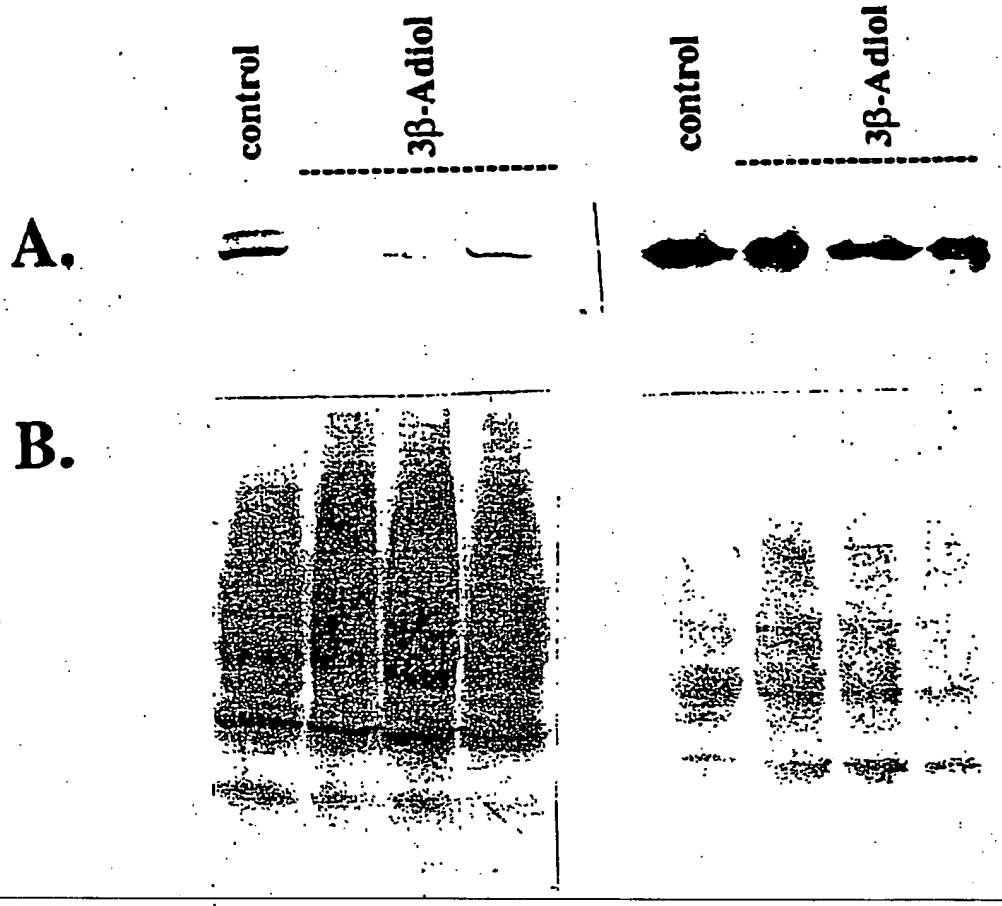

FIGURE 8

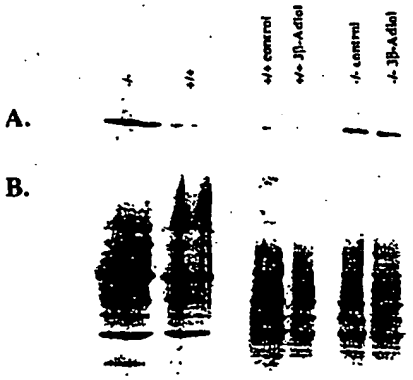

FIGURE 9

CANCER TREATMENT

Field of Invention

This invention relates to cancer treatment, particularly to the treatment of prostate cancer. The invention also relates to a method of regulating cancer growth in particular prostate cancer cell growth.

Discussion of Prior Art

Although there have been numerous attempts to find a correlation between estrogens and benign prostatic hyperplasia and/or prostatic cancer, no conclusive clear role for estrogens has been defined. Epidemiological and experimental studies indicate a dual role for estrogen in the development of prostate tumors, i.e. it is involved in both tumor promotion and chemoprevention (Olbina, G., *et al* (1998) *Prostate* **37**, 126-129; Martikainen, P. M., *et al* (1987) *Prostate* **11**, 291-303; Kawamura, H., *et al* (1993) *Anatomischer Anzeiger* **175**, 569-575; Shao, T. C. *et al* (1986) *Prostate* **8**, 349-362; Krieg, M., *et al* (1993) *Verhandlungen der Deutchschen Gesellschaft für Pathologie* **77**, 19-24). In men, the risk for benign prostatic hyperplasia (BPH) and prostatic cancer increases with age (Kozak, I., *et al* (1982) *Prostate* **3**, 433-438; Pylkkanen, L., *et al* (1996) *Eur. J. Urology* **30**, 243-248) and so do plasma levels of estrogens (17β-estradiol and estrone) and the ratio of estrogens to androgens.

Excessive exposure to estrogens during critical stages of development, or long-term treatment of adult animals with estrogens and androgens, promotes development of prostatic neoplasia Karr, J. P., *et al* (1984) *Urology* **23**, 276-289; Messina, M. J. *et al* (1994) *Nutrition & Cancer* **21**, 113-131). In an apparent direct contrast with this, epidemiological studies suggest that diets rich in phytoestrogens, particularly soy products, are associated with a low risk of prostate cancer (Barnes, S., *et al* (1995) *J. Cell Biochem* **22**, 181-177; Goodman, M. T., *et al* (1997) *Amer. J. Epidemiol.* **146**, 294-306) and have chemopreventive properties in experimental tumor models (Landstrom, M., *et al* (1998) *Prostate* **36**, 151-161; Hempstock, J., *et al* (1998) *Brit. J. Urology* **82**, 560-563). Some of these conflicting effects of estrogens may be explained by the fact that there are two distinct estrogen receptors which have unique and sometimes opposing roles in cells

(Paech, K., *et al* (1997) *Science* **277**, 1508-1510; Weihua, Z., *et al* (2000) *Proc. Natl. Acad. Sci. USA* **97**, 5936-5941).

The classical estrogen receptor, ERα, is present at low levels in prostatic stroma but is not detectable in the epithelium (Bodker, A., *et al* (1993) *Scand. J. Urology & Nephrol.* **27**, 169-174). Because of this, it had been assumed for a long time that the effects of estrogen on the prostatic epithelium were indirect (Kuiper, G. G. *et al* (1996) *Proc Natl Acad Sci USA* **93**, 5925-5930). The discovery of ERβ in the prostatic epithelium (Kuiper, G. G. *et al* (1996) *supra*) changed this view. Furthermore, developmental and hormonal regulation of ERβ mRNA (Chang, W. Y., *et al* (1999) *Endocrinology* **140**, 405-415; Prins, G. S., *et al* (1998) *Endocrinology* **139**, 874-883) suggests an important role for ERβ in regulation of prostatic growth.

Because estrogen disrupts the hypothalamic-pituitary-gonadal system, any direct effects on prostatic estrogen receptors, *in vivo*, can be masked by the effects of estrogen on the CNS-gonadal axis i.e. chemical castration. Direct effects of estrogen on the prostate have been demonstrated in castrated animals as well as in prostate organ cultures (Schneider, M. R., *et al* (1986) *J. Cancer Res. & Clin. Oncol.* **112**, 258-265; Lamartiniere, C. A., *et al* (1998) *Amer. J. Clin. Nutrition* **68**, 1400S-1405S). 5α-Androstane-3β,17β-diol (3βAdiol) is an estrogenic metabolite of 5α-dihydrotestosterone (DHT) which binds to both ERα and ERβ with a Kd of 1.9×10^{-8} M (Kuiper, G. G., *et al* (1997) Endocrinology **138**, 863-870). Its affinity for the androgen receptor is negligible (Turcotte, G., *et al* (1988) *J. Steroid Biochem.* **29**, 69-76). In the prostate, brain and pituitary, 3βAdiol is extensively metabolized into polar triols, which are hormonally inactive (Sundin, M., *et al* (1987) *J. Biol. Chem.* **262**, 12293-12297).

In human prostate cancer, expression of Ki-67 is correlated with tumor stage, Gleason score, and treatment failure (Khoo, V. S., *et al* (1999) *Prostate* **41**, 166-172; Janssen, T., *et al* (1995) *Prostate* **27**, 277-286). Despite strategies aimed at early detection and treatment, prostate cancer remains a leading cause of morbidity and mortality today. Current therapies have a limited impact on metastatic hormone-refractory cancer. One of the major problems in studying human cancers is availability of appropriate animal models. Cancer

cell lines have been extensively used in the study of mechanisms of hormone-induced cell proliferation (Zhao, X. Y., *et al* (1999) *J. Urology* 162, 2192-2199; Negri-Cesi, P., *et al* (1999) *Prostate* 41, 224-232), but clearly these cell lines are not ideal models of human organs.

The molecular mechanisms of action for ERα versus ERβ have recently been investigated. ERα and ERβ have almost identical DNA-binding domains and studies in vitro have demonstrated that the two receptors have similar affinities for estrogenic compounds (Kuiper, G. G. *et al* (1996) *Proc Natl Acad Sci USA* 93, 5925-5930, Kuiper, G. G., *et al* (1997) *supra*; Tremblay, G. B., *et al* (1997) *Mol Endocrinol* 11, 353-365). The amino-acid sequence of ERβ differs from ERα in the N- and C-terminal trans-activating regions. Therefore the transcriptional activation mediated by ERβ may be distinct from ERα (Paech. K., *et al* (1997) *supra*). Considering the great similarities in ligand- and DNA-binding specificity it has been speculated that a differential tissue distribution of estrogen receptors may be important for mediating tissue specific responses to estrogens (Kuiper, G. G., and Gustafsson, J. A. (1997) *FEBS Lett* 410, 87-90). Thus, the unique transactivating domains of the two receptor subtypes, in combination with differential tissue-distribution, or differential cell-type distribution within a tissue, could be important factors to determine the estrogen response in target tissues.

Mice lacking a functional ERα gene, ERα Knockout mice (ERKO), have been generated (Couse, J. F. *et al* (1995) *Mol. Endocrinol.* 9, 1441-1454) and more recently ERβ Knockout mice (BERKO) have also been described (Krege, J. H. et al (1998) *Proc. Natl. Acad. Sci. USA* 95, 15677-15682). Double-ER-Knockout mice (DERKO) i.e. mice having no estrogen receptors have also been generated.

The inventors have shown that ERβ protein is abundant in the nuclei of epithelial cells in the ventral prostates of rats and mice and that lack of ERβ in mice results in hyperplastic foci in the ventral prostates of young adult animals. Hyperplasia becomes more severe with age and by one year of age 80% of BERKO mice examined have dysplasia and PIN–like lesions in their ventral prostates. Epithelial cells in the ventral prostate of BERKO mice, are not at G_0 but are always in the cell cycle as indicated by the expression of the

proliferation cell antigen Ki-67 (Gerdes, J., *et al* (1984) *J. Immunol.* 133, 1710-1715).

The distinct patterns of cellular distribution of ERα and ERβ, in the ventral prostate, suggests that these two receptors have different roles in this organ. ERα is located in prostatic stromal tissue and is not detectable in the epithelium. ERβ is abundantly expressed in the epithelium where it is coexpressed with AR.

The inventors have shown that, on the basis of sedimentation profiles, the two estrogen receptors have distinct physical characteristics. ERβ sediments as a 4S peak regardless of the salt concentration. ERα sediments as a 4S peak in high salt (400 mM) and as an 8S peak in low salt (10 mM) (Inano, K., *et al* (1994) *supra*). Since the 8S ERα complex represents its association with heat shock proteins and other chaperones (Grossman, A. & Traish, A. (1992) *Life Sci.* 51, 859-867), it is likely that ERβ does not form the same types of complexes as does ERα. There have been several reports over the years of estrogen binding proteins in the liver, kidney, pancreas, uterus and oviduct (Tong, J. H., *et al* (1983) *J. Steroid Biochem.* 18, 273-279; McNaught, R. W. & Smith, R. G. (1986) *Biochemistry* 25, 2073-2081; Sultan, C., *et al* (1999) *Bulletin du Cancer* 86, 618-621), which sedimented at 4S in both high and low salt. The difference in sedimentation properties of the two receptors has provided a simple and sensitive method for measuring the relative amounts of ERα and ERβ in tissues and should prove useful for small biopsy samples from the clinics. This method complements immunoassays since it provides information about the binding properties of the receptors. The inventors have used sucrose gradient sedimentation to demonstrate that estradiol and 3βAdiol specifically successfully bound to and competed for binding to ERβ.

The epithelial AR appears to be one of the genes which is regulated by ERβ. The inventors have shown that there is elevated expression of the AR in BERKO mouse prostates and that the estrogen receptor ligand, 3βAdiol, downregulates AR in wild type but not in BERKO mice. Down regulation of AR could be one mechanism through which ERβ reduces or limits prostatic growth and induces a more differentiated prostate phenotype. Such a differentiating role for ERβ was suggested but not actually demonstrated by Prins *et*

al. (Chang, W. Y. *et al* (1999) *supra*; Prins, G. S. *et al* (1998) *supra*) on the basis of developmental studies of ERβ mRNA in the prostate. The extent to which elevation in AR levels contributes to BERKO prostate phenotype is unclear at present. Elevation of AR has been considered as one possible causative factor in development of prostate cancer (Gann, P. H., *et al* (1996) *J. Natl. Cancer Institute* **88**, 1118-1126). The precise relationship between prostatic androgen receptors, circulating androgen levels and prostate cancer remains poorly understood. Plasma levels of DHT are not elevated in prostate cancer patients (Gustafsson, O., *et al* (1996) *British J. Urology* **77**, 433-440; Vatten, L. J., *et al* (1997) *Cancer Epidemiology Biomarkers & Prevention* **6**, 967-969; McKenna, N. J., *et al* (1999) *Endocrine Rev.* **20**, 321-344). The inventors have shown that short term treatment with DHT induced Ki-67 in very few cells in the normal prostatic epithelium, a pattern quite different from the widespread expression seen in BERKO prostates. Overexpression of AR in cells cannot be equated with exposure to high levels of androgens. Overexpression of a nuclear receptor can lead to sequestration around that receptor of common nuclear receptor associated proteins such as corepressors or coactivators which could then cause disruption or dysregulation of other receptor pathways in the cell nucleus (Voigt, K. D. & Bartsch, W. (1986) *J. Steroid Biochem.* **25**, 749-757). If ERβ regulates prostatic growth, the physiological ligand for this receptor needs to be clearly identified. The concentration of estradiol in the prostate is low, approximately 0.1 pmol/g tissue (Bubendorf, L., *et al* (1998) *Human Pathology* **29**, 949-954). The prostatic concentration of 3βAdiol is 100-fold higher at 10 pmol/g (Bubendorft, L., *et al* (1998) *supra*) and 3βAdiol has been suggested previously to be an estrogen in the prostate (Bubendorft, L., *et al* (1998) *supra*). Since 3βAdiol is a metabolite of dihydrotestosterone its antiproliferative effects could well be a mechanism whereby the proliferative effects of DHT can be opposed. The inventors results on prostates from rats maintained on a soy-free diet revealed that there was estrogenic activity in the prostates. The majority of this activity co-eluted with 3βAdiol, not estradiol. If 3βAdiol is an endogenous ERβ ligand involved in limiting androgen-induced prostatic growth, it could be predicted that inhibitors of 5α-reductase would not be very effective in controlling prostatic growth since, in addition to blocking DHT formation, they would be removing the antiproliferative ligand, 3βAdiol.

The inventors have shown that BERKO mouse prostates display hyperplasia, dysplasia and PIN lesions as the animals age. In humans, prostate cancer develops in the outer regions of the prostate. There are no clearly defined prostatic lobes in the human prostate and it is difficult to make comparisons between the human and mouse prostate. However, some of the criteria suggested for a good animal model of human prostate cancer have been fulfilled by BERKO mice i.e. spontaneous occurrence, development with age of animal, prostatic in origin and slow growing adenocarcinoma. Other criteria such as, transplantability, metastatic potential and hormonal responsiveness may also be tested in the BERKO mouse.

In this invention an ERβ selective agonist is regarded as having a higher binding affinity or capacity to ERβ than ERα or other estrogen receptors, or have an exclusive binding affinity or capacity to ERβ.

Disclosure of the Invention

According to a first aspect of the invention there is provided the use of an ERβ agonist in the manufacture of a medicament for the treatment of cancer. The medicament is preferably for prostate cancer. The ERβ agonist may be selective for ERβ, such as genistein and 3βAdiol. ERβ-selective compounds are known. For example, WO98/56812 (Karo Bio AB) discloses certain ERα and ERβ selective ligands. Barkhem, T. *et al* discloses the selectivity of certain compounds for different forms of the estrogen receptor.

According to a second aspect of the invention there is provided a method of regulating prostatic epithelial growth and differentiation in a subject comprising treating the subject with an ERβ agonist. In this method the androgen receptor (AR) is downregulated.

According to a third aspect of the invention there is provided a method of screening compounds for use in the treatment of prostate cancer comprising the step of selecting compounds which bind ERβ, particularly those which are ERβ agonists. Preferably, the ERβ agonists are ERβ selective.

According to a fourth aspect of the invention there is provided a method of detecting the

onset of cancer in a subject comprising measuring the levels of androgen receptor in the subject. Again such a method may be suitable for prostate cancer.

According to a fifth aspect of the invention there is provided a pharmaceutical composition for the treatment of cancer, the composition comprising an ERβ agonist, which may be selective for ERβ. Such an agonist may be 3βAdiol, which may be in the dosage rangeof 0.1nM to 200nM, and preferably 0.1nM to 10nM.

Pharmaceutical compositions of this invention comprise any suitable ERβ agonists, and pharmaceutically acceptable salts thereof, with any pharmaceutically acceptable carrier, adjuvant or vehicle. Pharmaceutically acceptable carriers, adjuvants and vehicles that may be used in the pharmaceutical compositions of this invention include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene- polyoxypropylene-block polymers, polyethylene glycol and wool fat.

The pharmaceutical compositions of this invention may be administered orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally, vaginally or via an implanted reservoir. We prefer oral administration or administration by injection. The pharmaceutical compositions of this invention may contain any conventional non-toxic pharmaceutically-acceptable carriers, adjuvants or vehicles. The term parenteral as used herein includes subcutaneous, intracutaneous, intravenous, intramuscular, intra-articular, intrasynovial, intrasternal, intrathecal, intralesional and intracranial injection or infusion techniques.

The pharmaceutical compositions may be in the form of a sterile injectable preparation, for example, as a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to techniques known in the art using suitable dispersing or wetting

agents (such as, for example, Tween 80) and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are mannitol, water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or diglycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically-acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant such as Ph. Helv or a similar alcohol.

The pharmaceutical compositions of this invention may be orally administered in any orally acceptable dosage form including, but not limited to, capsules, tablets, and aqueous suspensions and solutions. In the case of tablets for oral use, carriers which are commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and dried corn starch. When aqueous suspensions are administered orally, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening and/or flavoring and/or coloring agents may be added.

The pharmaceutical compositions of this invention may also be administered in the form of suppositories for rectal administration. These compositions can be prepared by mixing a compound of this invention with a suitable non- irritating excipient which is solid at room temperature but liquid at the rectal temperature and therefore will melt in the rectum to release the active components. Such materials include, but are not limited to, cocoa butter, beeswax and polyethylene glycols.

Topical administration of the pharmaceutical compositions of this invention is especially useful when the desired treatment involves areas or organs readily accessible by topical application. For application topically to the skin, the pharmaceutical composition should

be formulated with a suitable ointment containing the active components suspended or dissolved in a carrier. Carriers for topical administration of the compounds of this invention include, but are not limited to, mineral oil, liquid petroleum, white petroleum, propylene glycol, polyoxyethylene polyoxypropylene compound, emulsifying wax and water. Alternatively, the pharmaceutical composition can be formulated with a suitable lotion or cream containing the active compound suspended or dissolved in a carrier. Suitable carriers include, but are not limited to, mineral oil, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water. The pharmaceutical compositions of this invention may also be topically applied to the lower intestinal tract by rectal suppository formulation or in a suitable enema formulation. Topically-transdermal patches are also included in this invention.

The pharmaceutical compositions of this invention may be administered by nasal aerosol or inhalation. Such compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilizing or dispersing agents known in the art.

According to a sixth aspect of the invention there is provided an ERβ agonist for use as a treatment for cancer, particularly prostate cancer. Preferably, the agonist may be selective for ERβ.

According to an seventh aspect of the invention there is provided the use of a BERKO mouse in the study of human prostate cancer. Preferably, the BERKO mouse displays at least one of hyperplasia and dysplasia and PIN lesions. The cells, tissues and other organs of the BERKO mouse may also be used.

The inventors have reasoned that by choosing an appropriate dose of 3βAdiol it is possible to obtain direct estrogenic effects in the prostate without disruption of pituitary function. Furthermore, since the effects of 3βAdiol are mediated by ERβ, comparison of BERKO

and +/+ mice regarding the responsiveness of their prostates to 3βAdiol, will provide information on the role of ERβ in the prostate.

Immunohistochemistry with specific ERβ antibodies revealed that the nuclei of more than 90% of prostatic epithelial cells of normal rats and mice harbour ERβ. By N-terminal amino acid sequencing of high salt extracts of mouse prostates, 549 and 530 molecular weight isoforms of ERβ were identified. With a combination of sucrose density gradient centrifugation and Western blotting , ERβ sedimented as a 4S peak in low salt extracts of the prostate and both estradiol or 5α-androstane-3β, 17 β-diol (3βAdiol) were good ligands. Morphological examination of 5-month old ERβ -/- (BERKO) mice revealed multiple hyperplastic foci in their ventral prostates. At one year of age lesions corresponding to low-grade prostatic intraepithelial neoplasia (PIN I and II) were seen. There were also different degrees of atypical adenomatous hyperplasia with nuclear dysplasia and pleomorphia. Surprisingly, in BERKO mouse prostates most of the epithelial cells expressed the proliferation antigen Ki-67. In wild type littermates (+/+) only occasional epithelial cells expressed Ki-67 and the epithelium was a single layer with no evidence of hyperplasia. Androgen receptor (AR) levels were higher in prostates of ERβ -/- mice than in those of +/+ littermates. Furthermore, 3βAdiol, an ERβ agonist down regulated AR in the prostates of +/+ but not -/- mice.

Brief Description of Drawings

Specific aspects of the invention will now be illustrated by way of example only with reference to the accompanying drawings Figures 1 to 9 in which:

Figure 1 shows a photomicrograph of immunohistochemical localization of ERβ in ventral prostates of rats and mice;

Figure 2 shows graphs a sucrose density gradient sedimentation profiles of ERα and ERβ;

Figure 3 shows photographs of gels of western blots showing ERβ in fractions from sucrose density gradient of 9-week-old rat prostates;

Figure 4 shows photographs of gels of Western blots showing the effects of estradiol and 3βAdiol in pitutary and aorta of intact and castrated male rat;

Figure 5 shows graphical representation of HPLC separation of 3βAdiol, 17β-estradiol and testosterone;

Figure 6 shows photomicrographs of the histology of ventral prostates from wild type and BERKO mice;

Figure 7 shows photomicrographs of the histology Ki-67 staining of ventral prostates wild-type and BERKO mice;

Figure 8 shows a photograph of gels of Western blots of androgen receptor (AR) and progesterone receptor (PR) from ventral prostates of 9 week old rats when treated with 3βAdiol; and

Figure 9 shows a photograph of gels of Western blots of AR levels in ventral prostates of wild type and BERKO mice.

Detailed Description of the Invention

Materials and Methods

A. **Animals:** Male Sprague-Dawley rats (6-12 week-old) and 9-week-old rats which were castrated at 6 weeks of age, were purchased from Mollegaard-Bommice (Ejby, Denmark). BERKOs, and their wild type littermates were housed in the transgenic facility at Huddinge hospital. Monthly checks were done for standard mouse pathogens by the Swedish Veterinary Association. Our mice did have pasteurella which is endemic in most mouse colonies in this country. All the animals were housed in a controlled environment on an illumination schedule of 12 h light/12 h dark, fed a standard pellet diet (containing soy meal) and water was provided *ad libitum*. 5α-Androstane-3β,17β-diol (200 mg/kg/day) or estradiol (1 mg/kg/day) dissolved in Intralipid (Pharmacia) were given daily to animals by s.c. injection. Control animals were given equal volumes of vehicle. After 7 days of treatment, the animals were

asphyxiated by CO_2 and ventral prostates, hearts, aorta and pituitaries were collected and either placed in appropriate fixatives or immediately frozen in liquid nitrogen for protein analysis. One s.c. injection of dihydrotesto-sterone (DHT)(1mg/kg) was given to the wild type littermates for Ki-67 immunostaining positive control, and tissues were collected 24 hours later.

B. Chemicals and antibodies: 5α-Androstane-3β,17β-diol, 5α-dihydrotestosterone, and 17b-estradiol were purchased from Sigma, [^{3}H] 17b-estradiol (48 Ci/mmol) from New England Nuclear, and [^{3}H]5α-androstane-3β,17β-diol (52.2 Ci/mmol) from Amersham. Polyclonal rabbit anti-androgen receptor (PA1-111A, rabbit) was from Affinity Bioreagents. Anti-progesterone receptor polyclonal antibody (PRc-19, rabbit) was from Santa Cruz. Anti-Hsp25 was from StressGen. Anti-lysozyme antibody was a gift from Dr. Giannis Spyrou (CBT, NOVUM, KI).

C. Antibody preparation and testing: Two antibodies which recognize ERβ and not ERα, have been prepared in this laboratory. LBD IgG was raised in rabbits with the ligand binding domain (LBD) of ERβ as an antigen. The ERβ LBD was expressed in SF9 cells and purified to homogeneity. IgG was prepared from the antiserum with protein A Sepharose chromatography. This IgG recognizes ERβ but not ERα on Western blots and the signal can be completely eliminated by preincubation of the IgG with LBD. Both ERα and ERβ were expressed in SF9 cells and used as standards. The second antibody was raised in chickens and purified from egg yolks. The antigen was full length ERβ expressed in SF9 cells. This IgY is useful in immunohistochemistry but does not give as strong signals as the LBD antibody on Western blots. Both antibodies recognize ERβ purchased from Panvera. Signals were eliminated by preadsorption of the IgY with ERβ protein purchased from Panvera or supplied by KaroBio, when these antigens were coupled to activated Sepharose. Preadsorption with the freely soluble antigens was much less effective.

D. Immunohistochemistry: For ERβ immunohistochemistry, tissues were removed immediately after sacrifice, frozen in liquid nitrogen and stored at -70°C. Frozen 8 mm sections were mounted on organosilane-coated slides, air dried for 30 min, fixed with

ice-cold methanol (3 min) and acetone (3 min) , air dried for 30 min, and stored at -20° C. After thawing, sections were fixed in 4% PFA for 10 min and rinsed with PBS. To quench endogenous peroxidase, slides were incubated with 0.5% H_2O_2 and to block unspecific binding of secondary antibody, sections were blocked with 10% rabbit serum. Sections were incubated with primary antibody (chicken IgY against ERβ) diluted 1:1000 in PBS with 3% BSA overnight at 4°C. Negative controls were incubated with 3%BSA in PBS, or ERβ antibody preadsorbed with ERβ coupled Sepharose. Slides were washed with PBS and incubated with secondary antibody (peroxidase conjugated rabbit anti chicken IgG, Sigma diluted 1:1000 in PBS) for 1h at room temperature. After thorough washing in PBS, sections were developed with DAB substrate (Zymed), lightly counterstained with Mauer's hematoxylin, dehydrated and mounted with permount. Ki-67 stainings were done according to the protocols described previously (Bodker, A., *et al* (1993) *Scand. J. Urology & Nephrol.* **27**, 169-174).

E. **Preadsorption of antibody:** ERβ 503 IgY was incubated with ERβ protein coupled to activated Sepharose for 12h at 4°C. ERβ protein used was either purchased from Panvera (Madison, WI, USA) or was the 503 protein which was used as antigen. As a control, bovine serum albumin coupled to Sepharose was also used for preadsorption.

F. **Histology:** For histological evaluation, ventral prostates were dissected apart under microscope. They were either fixed overnight in 4% PFA and processed routinely for paraffin embedding, or were frozen in liquid nitrogen. Paraffin or frozen sections were stained with hemotoxylin and eosin and evaluated under light microscope.

G. **Preparation of cytosol for sucrose density gradient centrifugation:** Tissue, frozen in liquid nitrogen, was pulverized in a dismembrator (Braun Melsungen) for 45 sec at 1800 RPM. Pulverized tissue was added to a buffer composed of 10 mM Tris chloride, pH 7.5, 1.5 mM EDTA and 5 mM sodium molybdate. For MCF-7 cells the suspension was 100 mg and for prostate it was 1g tissue per ml buffer. Cytosol was obtained by centrifugation of the homogenate at 204, 000 x g for 1 h in a 70Ti rotor at 4°C.

H. **Sucrose density gradient assay:** Gradients were done as previously described (Inano, K., *et al* (1994) *J. Biochem* 116, 759-766). Prostate extracts were incubated for 3 h at 0 °C with 10 nM [^{3}H]estradiol or [^{3}H] 3βAdiol, either in the presence or absence of an excess of radioinert estradiol and the bound and unbound steroids were separated with dextran-coated charcoal. Sucrose density gradients [10-30% (w/v) sucrose] were prepared in a buffer containing 10 mM Tris-HCl, 1.5 mM EDTA, 1 mM a-monothioglycerol (Sigma), 10 mM KCl. Samples of 200 ml were layered on 3.5 ml gradients and centrifuged for 16 h at 300,000 x g in an SW-60Ti rotor (Beckman Instruments, Palo Alto, CA) at 4 °C in a Beckman L-70K ultracentrifuge. Successive 100 ml fractions were collected from the bottom by paraffin oil displacement, using a collector of our own design, and assayed for radioactivity by liquid scintillation counting. For Western blotting, fractions were first precipitated with TCA, and the precipitate resuspended in methanol. Samples were placed on dry ice for 30 min and the protein recovered by centrifugation. Pellets were dissolved in SDS sample buffer and proteins resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in gradient gels 4-20%.

I. **Protein extraction for Western blotting:** All tissue handling was done at 4°C. Tissues were put into homogenization buffer (600 mM Tris-HCl, 1 mM EDTA, pH7.4) containing 2 protease inhibitor cocktail tablets (Boehringer) per 50 ml and homogenized for a few seconds using Polytron PT3100. The homogenates were then centrifuged for 1 h at 105,000xg to obtain the supernatant as tissue extracts. The protein contents of the tissue extracts were measured using Bio-Rad Protein Assay with BSA as the standard. This extraction procedure was used to compare the protein profiles in prostates from BERKO and wild type mice and 3βAdiol-treated rats and mice.

J. **Western blotting analysis:** Proteins were resolved on SDS polyacrylamide gels, 9% polyacrylamide or premade gradient gels, 4-20% (NOVEX), with a tris-glycine buffer system. Transfer to PVDF membranes was either by semi dry blotting or in a

tris-glycine buffer.

K. **N-terminal sequencing of proteins:** In order to obtain enough material for N-terminal amino acid sequencing of ERβ, cytosol was prepared from 13 g of rat ventral prostate. The cytosol was diluted 10-fold with 20 mM sodium phosphate buffer, pH 7.4 to reduce the salt concentration. Heparin-Sepharose (1ml) was added and the mixture gently rotated for 1h at 5°C. Heparin-Sepharose was recovered by centrifugation and washed 5 times with 20 mM sodium phosphate buffer. Proteins were eluted with 1M NaCl, precipitated with 10% TCA, washed with methanol and resolved on SDS gels in 6 lanes. Proteins were transferred to PVDF membranes, a strip was cut from one lane for detection of ERβ by Western blotting and the rest of the membrane was stained with Coomassie Brilliant blue. Protein bands corresponding to those which reacted with the LBD antibody were cut from the membrane and N-terminal sequencing performed on an Applied Biosystems 473A protein sequencer.

L. **Measurement of estrogen receptor activators in rat prostate:** Rats used in this experiment were from a colony raised on a soy-free diet. Prostates from 5-month old rats were homogenized in saline and the homogenates extracted three times with ethyl acetate. The extract was dried under a stream of nitrogen and the residue dissolved in ethanol. The ethanol soluble extract was analysed by HPLC on a C18 column) (4.6 mmx150 mm, Agitero Genotechnologies) on a Water HPLC system with a diode array detector. A solvent system of acteonitrile:water was used with the following acetonitrile concentrations: 1-2min 0%; 2-3 min 0-40%;3-33 min, 40-60%, 33-34 min 60-100%. Dried fractions from the HPLC eluate were dissolved in 50µl 70% ethanol and analyzed for estrogen/antiestrogen activity using a cell-based estrogen receptor-dependent transcription assay described previously (Barkhem, T., *et al* (1998) *Mol. Pharmocol* 54, 105-112). Briefly, 25 x 10^3 293/hERβ_{485} reporter cells per well were seeded in 96-well culture plates in 100 ml of Coon's/F12 (w/o phenol-red) supplemented with 10% FCS (stripped twice using dextran coated charcoal), 2 mM L-glutamine. 24h later conditioned medium was replaced with 100 ml Coon's/F12 supplemented with 1% FCS (estrogen depleted), 2 mM L-glutamine, gentamicin (50 mg/ml) and 1µl extract (1:50th of the extracts) in the absence or presence of 0.5 nM

17β-estradiol. The level of alkaline phosphatase reporter protein expressed from the DERE2-ALP reporter was determined by a chemiluminescent assay as described (Barkhem, T., *et al* (1998) *supra*). In all experiments cells were exposed to 0.1% ethanol, extracts and/or 17β-estradiol for 72 h before harvest and analysis for effect on reporter gene expression. Each extract was tested for activity in triplicate.

Results

1. **Immunohistochemical localization of ERβ in the mouse and rat ventral prostate**

   Frozen sections of ventral prostates from mice (a and b) and rats (c and d) were stained for ERβ with the chicken anti ERβ 503 IgY. Visualization was with DAB. Positive immunoreaction was indicated by brown color and was marked by arrows. Stromal cell nuclei, and some epithelial cell nuclei which were negative for ERβ, were evident by the counterstain color (blue). In adult mice and rats, the majority of epithelial cell nuclei in the ventral prostate stained positively for ERβ. There was no cytoplasmic staining. Stromal cells were mostly negative (Fig. 1). Preadsorption of the antibody with ERβ 503 or with ERβ completely abolished the nuclear staining.

2. **Identification of ERβ in prostate cytosols of rats and mice by sucrose density gradients**

   As previously described (Jensen, E. V., *et al* (1968) *Proc. Natl. Acad. Sci. USA* 59, 632-638), ERα in low salt extracts of rat uterine cytosol as well as cytosol from MCF7 cells binds tritium-labeled estradiol and sediments as an 8S peak. As shown in Fig. 2A, an 8S sedimentation peak was seen with cytosolic extracts of MCF7 cells and uterine cytosol but the cytosol of SF9 cells, which were over expressing ERβ, showed a sedimentation peak at 4S. In rat ventral prostate cytosols, binding of both [^{3}H]-estradiol and [^{3}H]-3βAdiol was observed only as 4S peaks. In panel A, the sources of ERα were cytosols from MCF7 cells (♦) and uteri of ovariectomized rats ( ). ERβ was prepared from extracts of SF9 cells expressing ERβ (o). In panel B, prostate cytosols were from rats which were 6 (o), 8 (Ê), 10 (•), and 12 ( ) weeks of age. In panel C, prostate cytosol from 9-week-old rats was used. The ligands were 5

nM [^{3}H]-3β Adiol (×) and [^{3}H]-3β Adiol plus radio inert 17β-estradiol, both at 5 nM (o). In panel D, prostate cytosol from 9-week-old rats was used. The ligands were 5 nM [^{3}H]-3β Adiol (×) and [^{3}H]-3β Adiol plus radio inert DHT, both at 5 nM (o). Proteins in every fourth fraction from the gradient depicted in Fig. 2 panel C, were precipitated and resolved on SDS gradient gels (4-20%). Proteins were transferred to PVDF membranes and probed with ERβ LBD antibody. Fig 3b is a duplicate of the blot in 3a, except that the primary antibody was preadsorbed with ERβ protein prior to Western blotting. Secondary antibodies were conjugated with horseradish peroxidase and signals were visualized by ECL.

Sedimentation of the estradiol-binding proteins in prostate cytosols from 6-, 8-, 10-, and 12-week-old rats (Fig. 2B) revealed that, when similar amounts of protein were loaded onto the gradients, binding in the 4S peak was almost undetectable at 6 weeks of age. By 8 weeks it was 60 fmol, and binding remained at this level in 10- and 12-week-old rats. Unlabeled estradiol (5 nM) competed with [^{3}H]-3βAdiol for binding in the 4S peak (Fig. 2C), confirming that binding was to an estrogen receptor and not to the androgen receptor. In Fig 2D, equal amounts of unlabeled DHT and [^{3}H]-3βAdiol (5 nM) were added and no competition was observed between DHT and [^{3}H]-3βAdiol, providing further evidence for the lack of involvement of AR in the 3βAdiol binding in prostate extracts. In +/+ mice at 5 months of age, with a high salt extract of prostates, a 4S estradiol binding peak was detectable at a level of 40 fmol/mg protein. In order to confirm that the binding in the 4S binding peak in low salt sucrose gradient assay was due to ERβ, fractions from the sucrose gradient of rat ventral prostates were analysed by Western blotting. A specific ERβ protein band of molecular mass 63 kDa coincided with the estradiol-binding peak on sucrose gradients (Fig. 3A). The specificity of the band was confirmed by preadsorption of the primary antibody with ERβ protein (Fig. 3B).

3. **Identification of prostatic ERβ by N-terminal sequencing.**

DNA-binding proteins were extracted from ventral prostates of 12-week old rats with the use of heparin-Sepharose. When this extract was probed with LBD antibodies on Western blots, a doublet of molecular weight 62-63 kD reacted with the antibody (data

not shown). Protein bands corresponding to this doublet were excised from the membrane and their N-terminals sequenced by automated Edman degradation. The amino acid sequences MEIKNSPSSLSSPA and MSIXXASSHKEFSQLR were read from protein bands of molecular mass 62-63 kDa. These two sequences correspond to ERβ containing 530 and 549 amino acids, respectively. There was no immunoreactive band at 55 kDa which is the expected size of the short form of the receptor containing 485 amino acids.

4. **Tissue specificity of 3βAdiol as an estrogen in rats**

Specific antibodies raised against lysozyme, Hsp27 and PR, were used to determine whether 3βAdiol was equally estrogenic in all tissues. The aorta and pituitary gland were chosen as known estrogen-responsive tissues. Western blots with total cellular extracts revealed that both estradiol and 3βAdiol induced lysozyme in the aorta (Fig. 4A). Estrogenic effects were more pronounced in rats three weeks after castration than in intact rats. In the pituitary gland (Fig. 4B, C), estrogen induced progesterone receptor and HSP27 but 3βAdiol had no effect on the expression of these proteins. Lysozyme (A) was measured in the aorta and progesterone receptor (B) and Hsp27 (C) were examined in pituitary. The control lanes of A and B represent data obtained by pooling of tissue from 4 mice. In the treatment groups, pituitaries from three individual mice were used. In panel A, data from both intact and castrated rats are shown because estrogen action on the aorta is more evident in castrated animals. Lysozyme and Hsp27 were measured in total cell extracts and PR in cytosol.

5. **Identification of 3βAdiol as an endogenous estrogen in the rat prostate.**

Rats used in this experiment were from a colony raised on a soy-free diet and have been found to have no measurable levels of phytoestrogens in their urine. Prostate extracts from 5-month old rats could activate estrogen receptors in a reporter assay. Upon HPLC resolution of the components of the extract, most of the estrogenic activity was found in a peak which eluted from the C18 column much earlier the than did estradiol (Fig 5). The peak coincided with the elution of 3βAdiol. There was some estrogenic activity in the fraction where estradiol is expected to elute but the estrogenicity in this fraction was 4 fold lower than that in the 3βAdiol peak (Fig. 5).

Panel A shows the HPLC seperation of standards, 3β Adiol, genistein, 17β-estradiol, and testosterone. 3β Adiol and genistein elute together at 12 mins. Estradiol elutes at 17 mins and testosterone after 18 mins. Panel B shows the estrogenic activity in HPLC fractions obtained from ethylacetate extracts of ventral prostates. Prostates were from rats fed a soy free diet from birth. Ethylacetate extracts of ventral prostates were analysed under conditions shown in panel a. Fractions were taken every 30 sec between 11.5 and 12.5 min, and 16.5 to 18.5 mins. 1:50th of the fractions (dissolved in 70% ethanol) were applied to reporter cells expressing human $ER\beta_{485}$. The expression of the stable integrated human placental alkaline phosphatase reporter gene is regulated by a single consensus estrogen response element. Cells were exposed to a final concentration of 0.1% ethanol and 1 µl of the indicated fraction +/- 0.5 nM 17β-estradiol (E2). Control cells were exposed to 0.1% ethanol only or 0.1% ethanol + 0.5 nM E2. Values are the mean from triplicate determinations, with error bars indicated for each value. For more details of the assay procedure see "Materials and Methods".

6. **Histology of normal and BERKO prostates**

At five months of age, in +/+ mice, the ventral prostates showed the normal secretory appearance with single-layer columnar or cuboidal epithelium and large lumina (Fig. 6A). In intact BERKO mice, there were increased infoldings of the epithelium and foci of epithelial hyperplasia (Fig. 6B and C) which were not present in all acinae. Hyperplastic foci were not found in +/+ littermates. Higer magnification of each section is inserted in the upper corner of each picture. Fig 6A is a section from the peripheral zone of a wild type mouse with normal infolding of the epithelium. Panel B (peripheral zone) and C (central zone) depict sections from BERKO mouse prostates. Hyperplastic foci in the BERKO prostate are indicated with arrows. Bars=50 µm.

At one year of age 8 out of 10 BERKO mouse prostates showed multiple lesions resembling the morphology of prostatic intraepithelial neoplasia (PIN lesions). Representative sections are shown in Fig. 7B to 7D (PIN grade I and II). and different degrees of atypical adenomatous hyperplasia with moderate nuclear dysplasia and proliferation forming cribriform patterns or "roman bridges" resembling what is seen

in clinging carcinoma of human breast (Quinn, C. M. & Ostrowski, J. L. (1997) *J. Clin. Pathol.* 50, 596-599). Some of the atypical epithelial cells showed prominent nucleoli and nuclear pleomorphia was also present. One-year-old wild type mose prostates were completely normal (Fig. 7A). Ventral prostates of wild type mice show a single layer of epithelial cells and no sign of hyperplasia (A). Ki-67 positive staining is rare (E). Ventral prostates of BERKO mice at one year of age show various degrees of atypical epithelial hyperplasia (B to D) resembling PIN lesions (C and D). Most of the epithelial cells in BERKO mouse prostates are Ki-67 positive (b and c). In panel (a) is the Ki-67 staining of and untreated wild type mouse and in (d) that of a wild type mouse after DHT treatment (1mg/kg for one day).

7. Ki-67 in the mouse prostate

At one year of age, Ki-67 positive cells are rare in the nuclei of prostatic epithelial cells of wild type mice, while most of the epithelial cells were Ki-67 positive in the BERKO prostate (Fig. 7E to G). Treatment of normal mice with DHT (1mg/kg for one day) resulted in induction of Ki-67 in prostate epithelium but this induction occurred in very few cells (Fig. 7H).

8. Detection of androgen receptor (AR) in wild type and BERKO prostates

AR was easily detected on Western blots with prostate cytosol of normal rats and mice. The immunoreactive protein migrated as a 114kDa protein. When cytosols were prepared from prostates of rats which were treated with 3βAdiol (200 mg/kg/day for 7 days), there was a reduction in the intensity of the AR signal (Fig. 8A). Equal amounts of cytosolic protein were loaded onto the lanes and this was confirmed by Coomassie staining of the membrane after Western blotting. When duplicate blots were probed for PR, there was no detectable difference in the intensity of the PR signal between the untreated and the 3βAdiol-treated rats (Fig. 8B). Panel a, shows Western blots with antibodies specific for AR (left side) and PR (right side). In panel b, membranes were stained with Coomassie Brilliant Blue after Western blotting. In each lane, 80 μg cytosolic protein was loaded. The control sample is an aliquot obtained by pooling prostates from three animals. In the 3βAdiol-treated group three individual prostates were analysed.

The level of expression of AR was higher in BERKO prostates than in prostates of +/+ littermates (Fig. 9A). In each lane, 100 μg of cytosolic protein, obtained by pooling the ventral prostates of 4 mice, was loaded. Panel A is a comparison of the androgen receptor content of untreated +/+ and -/- mice. In panel B , the effects of treatment of +/+ and -/- mice with 3βAdiol are illustrated. Blots were Coomassie stained after Western blotting to confirm equal loading of protein in the lanes. They are shown below their corresponding Western blots. In normal mice, as in rats, 3βAdiol-treatment caused a significant down regulation of AR (Fig. 9B). This was clearly evident on Western blots. No such reduction in AR level was detected in BERKO prostates upon treatment with 3βAdiol (Fig. 9C). Coomassie staining of the membrane after Western blotting confirmed that similar amounts of protein were loaded in each lane.

CLAIMS

1. Use of an ERβ agonist in the manufacture of a medicament for the treatment of cancer.

2. Use according to claim 1 wherein the ERβ agonist is selective for ERβ.

3. Use according to claim 1 or 2 wherein the cancer is prostate cancer.

4. Use according to any preceeding claim wherein the ERβ agonist is selected from genistein and 3βAdiol.

5. Use according to claim 4 wherein the agonist is 3βAdiol.

6. A method of regulating prostatic epithelial growth and differentiation in a subject comprising treating the subject with an ERβ agonist.

7. A method according to claim 6 wherein the ERβ agonist is selective for ERβ.

8. A method according to claim 6 or 7 wherein the agonist is selected from genistein and 3βAdiol.

9. A method according to claim 8 wherein the agonist is 3βAdiol.

10. A method according to any one of claims 6 to 9 wherein the androgen receptor is down regulated.

11. A method according to any one of claims 6 to 10 wherein the subject is human.

12. A method of screening compounds for use in the treatment of prostate cancer comprising the steps of selecting compounds which bind ER.

13. A method according to claim 12 wherein the selected compounds are selective for ERβ.

14. A method according to claim 13 wherein the selected compounds are ERβ agonist.

15. A method of detecting the onset of cancer in a subject comprising measuring the levels of androgen receptor in the subject.

16. A method according to claim 15 wherein the subject is a human.

17. A pharmaceutical composition for the treatment of cancer the composition comprising an ERβ agonist.

18. A composition according to claim 17 wherein the agonist is selective for ERβ.

19. A composition according to claim 17 or 18 wherein the ERβ agonist is selected from genistein and 3βAdiol.

20. A composition according to claim 19 wherein the agonist is 3βAdiol.

21. A composition according to claim 20 wherein the 3βAdiol is in the dosage range of 0.1nM to 200nM.

22. A composition according to claim 21 wherein the 3βAdiol is in the dosage range of 0.1nM to 10nM.

23. Use of a BERKO mouse in the study of human prostate cancer.

24. Use according to claim 23 wherein the BERKO mouse displays at least one of hyperplasia and dysplasia and PIN lesions.

25. Use according to claim 23 or 24 wherein the study is conducted on BERKO mouse cells, tissue or other organs.

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Application No:	GB 0026015.8	**Examiner:**	L.V.Thomas
Claims searched:	1-5 and 17-22	**Date of search:**	27 March 2001

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Databases searched:

UK Patent Office collections, including GB, EP, WO & US patent specifications, in:

UK Cl (Ed.S):

Int Cl (Ed.7):

Other: Online: EPODOC, WPI, BIOSIS, MEDLINE, CAS-ONLINE, SCISEARCH, EMBASE

Documents considered to be relevant:

Category	Identity of document and relevant passage		Relevant to claims
X	GB 2345851 A	(VINSON ET AL.) see p.1 ll.12-16, p.6 ll.3-10, p.7 ll.15-23 and claims 13-18	1-3,17,18
E, X	WO 00/62765 A2	(ASTRAZENECA) see p.1 ll.4-9, p.2 l.23 - p.3 l.20 and p.7 ll.19-29	1-3,17,18
X	WO 00/55137 A1	(SIGNAL PHARM. ET AL.) see p.6 ll.1-19, p.8 ll.18-24 and p.32 l.8 - p.33 l.28	1-3,17,18
X	WO 98/48790 A1	(ANTICANCER, INC.) see p.4 l.17 - p.5 l.27, p.9 l.25 - p.10 l.1, p.12 l.5 et seq. and Examples	1-4,17-19
A	Endocrinology 2000, 141(10), pp.3657-3667 "Resveratrol acts as a mixed .." - Bowers et al. - see abstract and p.3662 col.2 l.24 - p.3664 col.2 l.53		1
X	Oncogene 12 Oct. 2000, 19(43), pp.4970-4978 "Estrogen receptor β .." - Pettersson et al. - see pp.4972-4973 and p.4976 col.2		1,2,4, 17-19
X	Proc. Amer. Assoc. Cancer Res. 1999, 40, p.651 "Life-time genistein .." - Lamartiniere et al. - see abstract #4296		1-4,17-19

X Document indicating lack of novelty or inventive step	A Document indicating technological background and/or state of the art.
Y Document indicating lack of inventive step if combined with one or more other documents of same category.	P Document published on or after the declared priority date but before the filing date of this invention.
& Member of the same patent family	E Patent document published on or after, but with priority date earlier than, the filing date of this application.

Application No: GB 0026015.8 **Examiner:** L.V.Thomas
Claims searched: 1-5 and 17-22 **Date of search:** 27 March 2001

Category	Identity of document and relevant passage	Relevant to claims
X	Proc. Amer. Assoc. Cancer Res. 1997, 38, p.262 "Inhibition of human prostate .." - Bosland et al. - see abstract #1762	1-4,17-19
X	Nutrition & Cancer 1997, 27(1), pp.31-40 "Estrogenic and Anti-proliferative .." - Zava et al. - see abstract and pp.36-37	1,2,4, 17-19

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
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